Efficient Bioconversion of Echinocandin B to Its Nucleus by Overexpression of Deacylase Genes in Different Host Strains

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Anidulafungin, which noncompetitively inhibits β-(1,3)-d-glucan synthase in fungal cell wall biosynthesis, is the newest antifungal drug to be developed. Echinocandin B deacetylase from Actinoplanes utahensis NRRL 12052 catalyzes the cleavage of the linoleoyl group of echinocandin B, a key step in the process of manufacturing anidulafungin. Unfortunately, the natural yield of echinocandin B nucleus is low. In our study, the echinocandin B deacetylase gene was systematically overexpressed by genetic engineering in its original producer, A. utahensis, and in the heterologous hosts Streptomyces lividans TK24 and Streptomyces albus. The introduction of additional copies of the gene, under the control of PermE* or its native promoter, into hosts showed significant increases in its transcription level and in the efficiency of the bioconversion of echinocandin B to its nucleus. The conditions for the cultivation and bioconversion of A. utahensis have been optimized further to improve production. As a result, while the wild-type strain initially produced 0.36 g/liter, a concentration of 4.21 g/liter was obtained after the generation of a strain with additional copies of the gene and further optimization of the reaction conditions. These results are useful for enhancing echinocandin B nucleus production in A. utahensis. Our study could enable the engineering of commercially useful echinocandin B nucleus-overproducing stains.

Fungal infections are being seen in ever-increasing numbers, largely because of the increase in the size of the population at risk over the past 20 years. This population includes cancer patients, transplant recipients, and other individuals receiving immunosuppressive treatment. These people are at greater risk than others owing to their weakened immune systems and the chronic nature of diseases (1–3). The increased incidence of invasive fungal infections has created a major challenge for health care professionals. Since cell walls are present in fungal cells but absent in animal cells, the fungal cell wall perhaps represents the ideal target for the therapeutic treatment of fungal pathogens in humans (4, 5). The development of echinocandins, the first class of antifungals to target the fungal cell wall, was a milestone in antifungal chemotherapy (6, 7). Three semisynthetic echinocandin derivatives have been developed for clinical use: caspofungin, micafungin, and anidulafungin (8). Their strengths include low toxicity, rapid fungicidal activity against most isolates of Candida spp., and predictable, favorable kinetics allowing once-a-day dosing. Besides Candida spp., their inhibitory spectrum includes Aspergillus spp. and Pneumocystis jirovecii, but not Cryptococcus neoformans (9, 10).

Echinocandin B (ECB), obtained by the fermentation of Aspergillus nidulans and Aspergillus rugulosus, is known as one of the natural cyclic hexapeptides that have a linoleoyl side chain, which inhibits a crucial enzyme in fungal cell wall biosynthesis, β-(1,3)-d-glucan synthase (11). ECB can be modified by enzymatic deacylation to a cyclic hexapeptide without a linoleoyl side chain and by subsequent chemical reacylation to generate a few therapeutic antifungal agents for clinical practice, such as anidulafungin (12–15). A deacetylase from Actinoplanes utahensis NRRL 12052 catalyzes the cleavage of the linoleoyl side chain from ECB (Fig. 1), an essential reaction for the three subsequent synthetic steps (16). The enzyme is a membrane-associated heterodimer composed of 63-kDa and 18- to-20-kDa subunits, and the expression of its activity is not affected by any cofactors, metal ion chelators, or reducing agents. In addition to that of ECB, this deacetylase mediates the cleavage of acu-leacin A, FR901379, various semisynthetic ECB derivatives, daptomycin and its three derivatives, teicoplanin, pseudomycin A, and capsaicins (17, 18). Thus, it may become increasingly significant as a pharmaceutical biocatalyst.

However, enzymatic deacylation was rate-limiting when conducted with whole cells of A. utahensis. The low bioconversion yield was presumably related to inadequate production of the ECB deacetylase. Because of the need for improved antifungal agents for the treatment of systemic fungal infections and the broad specificity of the enzyme, we were interested in constructing engineered strains with high production of the enzyme and in developing a better bioconversion method to further improve the efficiency of bioconversion of ECB. Here we describe the cloning of the gene encoding ECB deacetylase, under the control of its native promoter or a PermE* promoter, into the original deacetylase-producing strain and two Streptomyces strains by Fc31-directed site-specific recombination in order to understand the effects of the promoters and gene dosage on the efficiency of the bioconversion of ECB to the ECB nucleus, particularly with regard to its potential biotechnological application.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and reagents.** The bacterial strains and plasmids used in this paper are listed in Table 1. Streptomyces lividans TK24, Streptomyces albus, and A. utahensis NRRL 12052 were obtained from our
laboratory. Biochemicals, chemicals, media, restriction enzymes, and other molecular biological reagents were from standard commercial sources.

**DNA isolation, manipulation, and sequencing.** DNA isolation and manipulation were performed by standard methods (21). PCR amplifications were conducted on an authorized Thermal Cycler (Eppendorf AG, Hamburg, Germany) using PrimerSTAR HS DNA polymerase (TaKaRa). Primer synthesis and DNA sequencing were carried out at Shanghai Invitrogen Biotechnology Co.

**Plasmid construction.** To express the ECB deacylase gene under the control of a *PermE* promoter, a 2.8-kb DNA fragment that contains only the gene encoding ECB deacylase was amplified by PCR from *A. utahensis* NRRL 12052 genomic DNA using primers 5'-AAAGAATTCTGCGGG CCTGAAA-3' and 5'-AAATCTAGAGACTGCGTGAGTTCTGC-3' and was cloned into the pSP72 vector, yielding pYG2001. The identity of the PCR product with the gene encoding ECB deacylase (GenBank accession number BD226911) was confirmed by sequencing. A 0.5-kb fragment containing a *PermE* promoter was PCR amplified from pYG1003 using primers 5'-AAAGAATTCTTACTAAGCCCCGACCCGAGCA-3' and 5'-A AAGAATTCTCCGGAGGTCGCAGC-3' and was cloned into the BglII/EcoRI site of pYG2001, yielding pYG2002. (Underlined letters in primer sequences represent restriction sites.) The 3.3-kb XbaI/XbaI fragment containing the gene coding for ECB deacylase and a *PermE* promoter from pYG2002 was inserted into the XbaI site of the pSET152 vector, yielding pYG2003 for the expression of the gene encoding ECB deacylase under the control of a *PermE* promoter by a *ΦC31*-directed site-specific recombination event.

For the expression of the gene encoding ECB deacylase under the control of its native promoter, a 4.0-kb DNA fragment that contains the gene encoding ECB deacylase with approximately 1 kb of upstream sequence and 0.5 kb of downstream sequence was amplified from *A. utahensis* NRRL 12052 genomic DNA by PCR using primers 5'-ATAGAATT CCGTGCCCAGCTGTTC-3' and 5'-AAATCTAGAGACTGCGTGAGTTCTGC-3'. Sequences represent restriction sites.) The 3.3-kb XbaI/XbaI fragment containing the gene coding for ECB deacylase and a *PermE* promoter was cloned into the pSET152 vector, yielding pYG2003 for the expression of the gene encoding ECB deacylase under the control of a *PermE* promoter by a *ΦC31*-directed site-specific recombination event.

**FIG 1** ECB deacylase-catalyzed reaction.

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**TABLE 1** Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Source or reference</th>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td><em>E. coli</em></td>
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<tr>
<td>DH5α</td>
<td>Host for general cloning</td>
<td>Invitrogen</td>
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<tr>
<td>ET12567(pUZ8002)</td>
<td>Donor strain for conjugation between <em>E. coli</em> and <em>Actinomyces</em></td>
<td>3</td>
</tr>
<tr>
<td>A. <em>utahensis</em></td>
<td>ECB deacylase-producing strain</td>
<td>NRRL 12052</td>
</tr>
<tr>
<td>DYG2001</td>
<td><em>A. utahensis</em> derivative with two copies of the ECB deacylase gene (second copy under the control of <em>PermE</em>&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>This work</td>
</tr>
<tr>
<td>DYG2002</td>
<td><em>A. utahensis</em> derivative with two copies of the ECB deacylase gene under the control of its native promoter</td>
<td>This work</td>
</tr>
<tr>
<td>DYG2003</td>
<td><em>A. utahensis</em> derivative with three copies of the ECB deacylase gene (second and third copies under the control of <em>PermE</em>&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>This work</td>
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<tr>
<td>S. <em>lividans</em></td>
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<tr>
<td>TK24</td>
<td><em>S. lividans</em> derivative with a single copy of the ECB deacylase gene under the control of <em>PermE</em>&lt;sup&gt;+&lt;/sup&gt;</td>
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</tr>
<tr>
<td>DYG2004</td>
<td><em>S. lividans</em> derivative with a single copy of the ECB deacylase gene under the control of its native promoter</td>
<td>This work</td>
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<td>DYG2006</td>
<td><em>S. lividans</em> derivative with two copies of the ECB deacylase gene under the control of <em>PermE</em>&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<td>S. <em>albus</em></td>
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<tr>
<td>DYG2007</td>
<td><em>S. albus</em> derivative with a single copy of the ECB deacylase gene under the control of <em>PermE</em>&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This work</td>
</tr>
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<td>DYG2008</td>
<td><em>S. albus</em> derivative with a single copy of the ECB deacylase gene under the control of its native promoter</td>
<td>This work</td>
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<tr>
<td>DYG2009</td>
<td><em>S. albus</em> derivative with two copies of the ECB deacylase gene under the control of <em>PermE</em>&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This work</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pSP72</td>
<td><em>E. coli</em> subcloning vector</td>
<td>Promega</td>
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<tr>
<td>pSET152</td>
<td><em>E. coli</em> replicon, Streptomyces <em>ΦC31</em> attachment site; Apr&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>pYG1003</td>
<td>2.0-kb fragment containing <em>PermE</em>&lt;sup&gt;+&lt;/sup&gt;-controlled aveBIV in pSET152</td>
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</tr>
<tr>
<td>pYG2001</td>
<td>2.8-kb PCR fragment containing the ECB deacylase gene in pSP72</td>
<td>This work</td>
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<tr>
<td>pYG2002</td>
<td>3.3-kb PCR fragment containing a <em>PermE</em>&lt;sup&gt;+&lt;/sup&gt;-controlled ECB deacylase gene in pSP72</td>
<td>This work</td>
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<tr>
<td>pYG2003</td>
<td>3.3-kb PCR fragment containing a <em>PermE</em>&lt;sup&gt;+&lt;/sup&gt;-controlled ECB deacylase gene in pSET152</td>
<td>This work</td>
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<tr>
<td>pYG2004</td>
<td>4.0-kb PCR fragment containing the ECB deacylase gene and its 5' and 3' regulatory sequences in pSP72</td>
<td>This work</td>
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<tr>
<td>pYG2005</td>
<td>4.0-kb PCR fragment containing the ECB deacylase gene and its 5' and 3' regulatory sequences in pSET152</td>
<td>This work</td>
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<tr>
<td>pYG2006</td>
<td>3.3-kb PCR fragment containing a <em>PermE</em>&lt;sup&gt;+&lt;/sup&gt;-controlled ECB deacylase gene in pSP72</td>
<td>This work</td>
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<tr>
<td>pYG2007</td>
<td>Two copies of a 3.3-kb PCR fragment containing a <em>PermE</em>&lt;sup&gt;+&lt;/sup&gt;-controlled ECB deacylase gene in pYG2003</td>
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TCTGC-3' and was cloned into the pSP72 vector, yielding pYG2004. The identity of the PCR product with the gene encoding ECB deacylase was also confirmed by sequencing. The 4.0-kb EcoRI/XbaI fragment from pYG2004 was inserted into the corresponding sites of pSET152, yielding pYG2005.

In order to express two copies of the gene encoding ECB deacylase under the control of a Perme promoter, another 3.3-kb DNA fragment with terminal EcoRI/EcoRV sites containing the gene encoding ECB deacylase and a Perme promoter was amplified by PCR from pYG2002 using primers 5'-AAAGATATCAGCCGCCCCAGGAGAATGC-3' and 5'-AAA GATATCAGGCTTGAGTCTGC-3' and was cloned into the pSP72 vector, yielding pYG2006. The identity of the PCR product with the gene encoding ECB deacylase was confirmed. The 3.3-kb EcoRI/EcoRV fragment containing the gene encoding ECB deacylase and a Perme promoter from pYG2006 was inserted into the corresponding site of the recombinant vector pYG2003, yielding the last vector, pYG2007, containing two deacylase gene expression cassettes. The correct directions of the gene encoding ECB deacylase and the Perme promoter in the pYG2003 or pYG2007 vector were verified by restriction enzyme digestion.

Conjugation and generation of recombinant strains. For overexpression and heterologous expression of the ECB deacylase gene in A. utahensis NRII 12052 and the two Streptomyces strains (S. lividans TK24 and the S. albus strain), expression vectors pYG2003, pYG2005, and pYG2007 were each introduced into hosts by intergeneric conjugation from Escherichia coli ET12567(pUZ8002) according to the standard procedure (19, 22). Transformants that were resistant to apramycin were identified as the recombinant strains, whose genomic DNAs were integrated with the deacylase gene and the apramycin resistance gene by FC31-directed site-specific recombination. The genotypes of the recombinant strains were further confirmed by PCR amplification with the vector-specific primer pair M13-47 and RV-M.

Culture growth and deacylation procedure. Wild-type and recombinant strains were grown on agar plates with a medium consisting of 2% soluble starch, 0.05% NaCl, 0.05% KH2PO4, 0.1% KNO3, 0.05% MgSO4·7H2O, 0.001% FeSO4·7H2O, and 2% agar powder (pH 7.4) at 28°C for sporulation. For the fermentation of A. utahensis NRII 12052, an agar piece around 1 cm2 was inoculated into a 250-ml flask containing 50 ml of a seed medium consisting of 2.5% sucrose, 2.0% oatmeal, 0.25% yeast powder, 0.1% KH2PO4, 0.05% KCL, 0.05% MgSO4·7H2O, and 0.0002% FeSO4·7H2O and was incubated at 28°C and 220 rpm for 3 days. A 250-ml flask containing 50 ml of fresh fermentation medium, consisting of 2% sucrose, 1% peanut meal, 0.1% KH2PO4, and 0.025% MgSO4·7H2O, was then inoculated with 5 ml of the seed culture, and fermentation was continued at 28°C and 220 rpm for 4 days. For the fermentation of S. lividans TK24 and S. albus, an agar piece around 1 cm2 was inoculated into a 250-ml flask containing 50 ml of a seed medium consisting of 1.0% glucose, 0.5% yeast powder, and 1% peptone and was incubated at 30°C and 220 rpm for 30 h. A 250-ml flask containing 50 ml of fresh fermentation medium consisting of 2.5% glucose, 1% bean flour, 0.3% NaCl, and 0.3% CaCO3 was then inoculated with 1 ml of the seed culture, and incubation was continued at 30°C and 220 rpm for 2 days.

The wet mycelia (15 g) were harvested by centrifugation, washed twice with double-distilled water, and then resuspended in a 50-ml biotransformation reaction mixture containing 0.1 M Na2HPO4/NaH2PO4 buffer (pH 6.8) in the presence of 2% dimethyl sulfoxide (DMSO). The enzymatic reaction was initiated by addition of the substrate ECB (1.5 g/liter) and was continued at 30°C and 220 rpm for 5 h before sample analysis.

Different experiments were subsequently conducted to optimize the cultivation medium of A. utahensis by changing the carbon and nitrogen sources and their proportions. Maltose, sucrose, glycerol, lactose, and soybean oil were used individually as the carbon source, at concentrations of 20 g/liter, instead of glucose. Cottonseed meal, soybean powder, and oatmeal were used individually as the nitrogen source, at concentrations of 10 g/liter, instead of peanut meal. The optimum concentration of sucrose or cottonseed meal was determined by dosing different amounts of sucrose (2 to 6%) or cottonseed meal (1 to 5%). The optimum pH for biotransformation was determined at 30°C. The optimum temperature for biotransformation was determined at pH 6.8. Ethanol was used as the cosolvent instead of DMSO. The optimum concentration of the ECB substrate was determined by dosing different amounts of ECB (1.5 to 12 g/liter) under the optimized conditions.

Analytical methods for the detection of ECB and the ECB nucleus. The reaction was interrupted by the addition of methanol. After low-speed centrifugation to remove precipitated proteins and mycelia, the activity of the deacylase was determined by monitoring the formation of the cyclic hexapeptide (the ECB nucleus) by high-performance liquid chromatography (HPLC) with an Agilent C18 column (250 by 4.6 mm; Agilent, Palo Alto, CA). The column was equilibrated with 92% solvent A (H2O containing 2 mg/ml ammonium acetate) and 8% solvent B (60% CH3CN containing 2 mg/ml ammonium acetate), and the analytical method was developed as follows: At 0 min to 15 min, a linear gradient from 92% A–8% B to 2% A–98% B; from 15 min to 25 min, a linear gradient from 2% A–98% B to 8% A–92% B; and from 25 min to 32 min, a constant proportion of 92% A–8% B. HPLC was carried out at a flow rate of 0.8 ml/min with UV detection at 222 nm using an Agilent 1120 HPLC system (Agilent Technologies, Palo Alto, CA). The identities of compounds were confirmed by liquid chromatography-mass spectrometry (LC-MS) analysis performed on an LCMS-2010A system (Shimadzu, Japan).

Assay of transcript levels by RT-PCR amplification. Total RNAs of three wild-type strains and nine recombinant strains were isolated from mycelia in the fermentation cultures. An additional purification step was carried out by using TRIzol reagent (catalog no. 15596-026; Invitrogen). To obtain cDNAs, DNase treatment and reverse transcription were performed by using random primers (catalog no. C1181; Promega) and Moloney murine leukemia virus (M-MLV) reverse transcriptase (catalog no. M1701; Promega) according to the manufacturer’s instructions. The transcript levels of the ECB deacylase gene were assayed on a Rotor-Gene RG-3000A instrument (Corbett Research Co., Australia), using real-time quantitative PCR and the 2−ΔΔCT method (23). Reverse transcription-PCR (RT-PCR) amplification was performed on each 25 µl of the mixture (consisting of 1 µg/ml of template cDNA, 2X SYBR Premix [Shanghai Xinghan Sci-Tech Co., Shanghai, China] and 0.4 µm forward and reverse primers) with the following program: 95°C for 3 min, followed by 30 cycles of 94°C for 20 s, 60°C for 20 s, and 72°C for 15 s. For assessment of the transcript level of the ECB deacylase gene, the 244-bp internal fragment was amplified from cDNA of the recombinant strains and wild-type A. utahensis NRII 12052 using primers 5'-CGGATATCTACGGAGGCATTGAC-3' and 5'-GCACAGCGGGAGCGGCTGA-3'. For assessment of the transcript level of the 16S RNA gene (as a control), the 242-bp internal fragments were amplified from cDNA of S. lividans TK24 and its recombinant strains using primers 5'-GCAATCTGGCTTCATCTCT-3' and 5'-ATGCGGCAACTGCTGCGCCTGC-3'; the 285-bp internal fragments were amplified from cDNA of S. albus and its recombinant strains using primers 5'-GGGAGCCGACGGTGGGATT-3' and 5'-GAGCCGCGGGATTTCACATT-3'.

RESULTS AND DISCUSSION

Qualitative and quantitative analysis of ECB and the ECB nucleus. ECB and its nucleus were extracted from a bioconversion mixture, and their identities were determined according to the method described above. As shown in Fig. 2, analysis of the samples revealed that the product and the remaining substrate ECB had the same retention times as those of the standard ECB nucleus (6.75 min) and ECB (22.80 min), respectively. Their identities were further confirmed by electrospray ionization-quadrupole time of flight (ESI-Q-TOF)–MS analysis, showing (M + Na)⁺ ions at m/z 820.40, consistent with the molecular formula...
C$_{34}$H$_{51}$N$_7$O$_{15}$ for the ECB nucleus, and (M + Na)$^+$ ions at m/z 1,082.61, consistent with the molecular formula C$_{52}$H$_{81}$N$_7$O$_{16}$ for ECB. The concentration of the ECB nucleus in the reaction mixture was calculated according to the standard curve of the reference ECB nucleus. The methods described here for qualitative and quantitative analysis of ECB and the ECB nucleus were applied to the biotransformation reaction mixture of A. utahensis, S. lividans, S. albus, and the recombinant strains obtained in this study.

**Overexpression of the ECB deacylase gene in A. utahensis NRRL 12052.** Enzymes can be overproduced by increasing the gene copy number. To investigate the effect of deacylase gene dosage, pYG2003 and pYG2007, which contain one and two deacylase gene expression cassettes, respectively, under the control of a PermE* promoter, were constructed in pSET152 and were introduced into A. utahensis NRRL 12052 by a ΦC31-directed site-specific recombination event, yielding recombinant strains DYG2001, containing two copies of the deacylase gene, and DYG2003, containing three copies of the deacylase gene. To express deacylase under the control of the native promoter, pTG2005 was constructed in pSET152 and was introduced into A. utahensis NRRL 12052, yielding recombinant strain DYG2002, which contained two copies of the deacylase gene (Fig. 3A). The 3.3-kb, 4.0-kb, and 6.6-kb fragments could be amplified separately from genomic DNAs of the recombinant strains mentioned above by PCR using primers M13-47 and RV-M. Sequencing showed that these recombinant strains had the designed genotypes (Fig. 4). Besides this, there were no apparent differences in growth characteristics or morphology.

Based on these results, we determined the bioconversion efficiency of each recombinant strain. As shown in Fig. 5, the efficiency of bioconversion of ECB was doubled in DYG2001 and DYG2002. These observations may mean that insufficient ECB deacylase activity was present in vivo. This idea is supported by other results. Even higher bioconversion efficiency was observed for DYG2003, containing three copies of the deacylase gene (Fig. 5). In
In our study, we found a decline in the level of the ECB substrate in the control system containing only the substrate and the bioconversion buffer, while the ECB nucleus product could be accumulated stably. In the *A. utahensis* NRRL 12052 bioconversion system, we found both the ECB nucleus product and the ECB substrate, while no visible ECB substrate was detected in the three recombinant strains upon HPLC analysis, as shown in Fig. 3B.

Another interesting question is the means of integrating the deacylase gene into *A. utahensis* and *Streptomyces*. As has been observed previously with 4'-epidaunorubicin production in *Streptomyces coeruleorubidus* (20), the ECB deacylase gene could be stably integrated into the genomes of host strains by a ΦC31-directed site-specific recombination event. Thus, it is not necessary to use a culture medium with apramycin resistance for the fermentation of the recombinant strains. Such a medium without apramycin helps cut the cost of ECB nucleus production on an industrial scale.

**Heterologous expression of the ECB deacylase gene controlled by different promoters.** *Streptomyces* spp. are well-known producers of biologically active compounds and enzymes (24–26). Techniques for the cultivation of these organisms on an industrial scale are well established, so these species are especially attractive as hosts for heterologous products (27). Different species of *Streptomyces* were screened as heterologous expression hosts by using the same bioconversion method as *A. utahensis*. For the two species examined, *S. lividans* (strain TK24) and *S. albus*, no ECB nucleus or ECB from whole cells was detected in the reaction mixtures. The *PermE* promoter has been used frequently as a strong constitutive promoter for native and heterologous genes in
Streptomyces species and related bacteria (28). To compare the expression levels of the ECB deacylase gene under the control of its native promoter and under the control of a PermE* promoter, the genes were cloned into pSET152 and were then transformed into the two Streptomyces species by a C31-directed site-specific recombination event to generate four new strains: DYG2004, DYG2005, DYG2007, and DYG2008. Another expression plasmid, pYG2007, with two ECB deacylase gene expression cassettes controlled by PermE*, was constructed and was introduced into S. lividans TK24 and S. albus, respectively, to yield DYG2006 and DYG2009, containing two copies of the deacylase gene (Fig. 3A). The genotypes of these recombinant strains were verified by PCR amplification, whereas no PCR products were isolated from the wild-type strains, as expected (Fig. 4). Subsequently, the bioconversion efficiencies of these recombinant strains were determined, and each reaction mixture was analyzed by HPLC. HPLC analysis revealed that the bioconversion efficiencies of these recombinant strains were enhanced over that of the original strain, A. utahensis NRRL 12052. More importantly, the bioconversion efficiencies of the recombinant strains DYG2004 and DYG2007, containing PermE*-controlled ECB deacylase, were higher than those of strains DYG2005 and DYG2008, in which the ECB deacylase gene was controlled by its native promoter. Strains DYG2006 and DYG2009, containing two copies of the deacylase gene, have higher bioconversion efficiencies than strains with one deacylase gene copy (Fig. 5). These findings demonstrated that PermE* was superior to the native promoter in the production of ECB deacylase for heterologous expression or overexpression and that there was potentially an enhancement of bioconversion efficiency and an improvement in the production of ECB deacylase due to the increase in gene copy number. As has been observed previously, the Streptomyces genome may contain multiple pseudo-attB sites. However, the frequency of integration of pSET152 into the ΔattB strain with additional pseudo-attB sites is reduced approximately 300-fold (29). In our study, there are strong positive correlations between the gene copy numbers of the recombinant strains and the transcript levels of the ECB deacylase gene. Therefore, there may be only one integrated gene. Of all the recombinant strains, DYG2003 from A. utahensis NRRL 12052 showed the highest bioconversion efficiency. A possible reason is that the ECB deacylase gene can be expressed better in A. utahensis NRRL 12052 and that DYG2003 contains more deacylase gene copies than NRRL 12052.

Optimal conditions for biotransformation of A. utahensis. For the cultivation of A. utahensis and its recombinant strains, the new fermentation medium formulation based on 3.0% sucrose, 2.0% cottonseed meal, 0.12% K2HPO4·3H2O, and 0.05% KH2PO4·3H2O was a good alternative solution for the pigmentation of strain DYG2003 (unpublished data). As a result, the growth rate and mycelium volume of A. utahensis and its recom-
binant strains increased dramatically. Under the new culture conditions, the concentration of mycelium in the fermentation broth was 30% higher than that under the original conditions in the same fermentation time. For the biotransformation reaction, the optimal temperature was 25°C and the optimal pH was 4.5 (Fig. 6A and B). The optimum concentration of ECB in the reaction system was detected by additions of different ECB quantities under the optimized conditions (pH 4 and 25°C) for further evaluation of the bioconversion efficiency of the recombinant strain DY2003 and the original strain A. utahensis NRRL 12052. With whole cells of NRRL 12052, the production of the ECB nucleus in reaction mixtures could rise as high as 4.21 g/liter when a suitable concentration of ECB (8 g/liter) was added to the reaction system (Fig. 6C). Additionally, ethanol is more economical and beneficial than DMSO as the cosolvent used in the reaction system to simplify the separation and purification of the product.

**ASSAY OF THE TRANSCRIPT LEVELS OF THE GENE BY RT-PCR AMPLIFICATION.** To assess the effects of different promoters and gene doses on the increase in the amount of enzyme produced, RT-PCR amplification for analysis of the transcription levels of the ECB deacylase gene under the control of promoters. The transcript levels of the gene encoding ECB deacylase leads to a significant increase in the transcript level of this gene, especially when it is controlled by the *Perme* promoter. The transcript levels of the gene meet the expectation of increased efficiency of bioconversion of ECB to the ECB nucleus in recombinant strains (mentioned above).

The deacylation of ECB is the starting point for the semisynthesis of anidulafungin. The current annual production of anidulafungin is on the order of tons, and anidulafungin is widely used as an important anti-infective drug. Synthesis of the ECB nucleus by chemical deacylation is extremely difficult, and the biotransformation method described here avoids this problem. The findings of this study could enable the engineering of commercially useful ECB nucleus-overproducing strains.

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